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## Effect of Calcium Glucarate on $\beta$ -Glucuronidase Activity and Glucarate Content of Certain Vegetables and Fruits

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Glucuronidation, a phase II conjugation reaction for many endogenous metabolites and xenobiotics, is catalyzed by glucuronosyltransferase (UDPgucuronate  $\beta$ -D-glucuronosyltransferase, UDPGT; EC 2.4.1.17). The opposite deglucuronidation reaction is catalyzed by  $\beta$ -glucuronidase ( $\beta$ -Gase; EC 3.2.1.31). D-Glucaro-1,4-lactone (GL) is a specific inhibitor of  $\beta$ -Gase (cf. (1)) and should theoretically enhance net glucuronidation in most tissues. Feeding CaG to experimental animals appears to increase the net glucuronidation of certain carcinogens and promoting agents, resulting in a marked inhibition of chemical carcinogen-mediated tumorigenesis (2-6). This activity of CaG is believed to be due to slow conversion of approximately one-third to the GL, a potent  $\beta$ -Gase inhibitor at the acid pH of the stomach (2-4,7). Dietary CaG increased the glucuronidation and excretion of bilirubin (2) and steroid hormones (3). Our recent studies (7) indicate that CaG interacts synergistically with retinoids to inhibit carcinogenesis in rodents. The potential impact of dietary glucarate may be very significant since a multitude of xenobiotics (including drugs and toxins) and endogenous metabolites are substrates or are metabolizable to substrates for glucuronidation.

Defining the modulating influence of dietary CaG on metabolism is important for at least two reasons. First there is, as noted above, accumulating evidence that it does have a significant modulating effect on metabolism of some compounds and consequent status of tissues or organism. Second, both glucarate and GL with which it is in equilibrium are normally present in low concentrations in the body as end products of glucuronate metabolism. Both glucarate and GL are also excreted in the urine (8). The present investigation further defines the effect of dietary glucarate on  $\beta$ -Gase in the tissues and blood. Because of the

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potential effect of deglucuronidation of glucuronides excreted in the bile by intestinal bacterial  $\beta$ -Gase on enterohepatic circulation (9, 10), the effect of glucarate ingestion on intestinal bacterial and mucosal  $\beta$ -Gase was also evaluated. Finally, the glucarate content of selected foods was analyzed to assess the potential impact of glucarate normally present in the diet.

## MATERIALS AND METHODS

### *Animals*

Adult female Fischer (F-344) strain rats (200-225g), purchased from Harlan Labs (Indianapolis, IN) were housed in a temperature ( $22 \pm 1^\circ\text{C}$ ), humidity (40-65%), and light (6:00 AM-6:00 PM) controlled room. The animals had access to food and water *ad libitum*. Food intake and weight gain were identical for rats on control (chow) and supplemented diets (7).

### *Effect of Acute Treatment of CaG on $\beta$ -Gase*

Two groups of five rats per group were fasted overnight before use. A single dose (4.5 mmole/kg body wt) of CaG (Gallard-Schlessinger Corp., New York, NY) suspended in 1 ml of 10% aqueous gum acacia was given to one group of rats by gavage at 10 AM. The second group, which received an equivalent amount of gum acacia, served as the control. Rats were anesthetized 4 hr after CaG administration with halothane, blood was collected by cardiac puncture, and then animals were sacrificed. Liver, lung, and gastrointestinal tract were isolated, washed with a 0.9% saline, and used for the preparation of microsomes for  $\beta$ -Gase assay. Serum was prepared from clotted blood by centrifugation at 1000g for 10 min.

### *Effect of Chronic Treatment of CaG on $\beta$ -Gase*

Two groups of adult female Fischer rats were used. One group was fed rodent chow (ProLab, Syracuse, NY) and served as control and the other group was fed rodent chow fortified with CaG (4%, w/w) for 2 weeks. As has been established earlier (2-7,19) food intake and weight gain of the rats was not affected by supplementation of the diet with CaG. Five animals were anesthetized using halothane for blood collecting, then sacrificed at either 2- or 4-hr intervals. Liver and intestines were obtained, washed with 0.9% saline, and used for the preparation of microsomes for  $\beta$ -Gase assay. Serum was collected by centrifuging the blood as described in the previous experiment. For the bacterial  $\beta$ -Gase assay, rats were sacrificed at 10 AM. The intestinal contents were diluted with 0.9% saline and centrifuged at 15,000g for 30 min. The pellet containing bacteria was suspended in distilled water and sonicated for 7 min and used for  $\beta$ -Gase assay (10).

In an earlier communication from our laboratories (3) we studied the effect of dietary supplementation of CaG (from 0 to 10%, w/w) on serum  $\beta$ -Gase activity. The results indicated that the degree of  $\beta$ -Gase inhibition by CaG remains about the same at the concentration from 4-10% dietary supplementation. Therefore, in this study we designed the experiment with only 4% dietary CaG supple-

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mentation. CaG at dietary concentrations below 4% produced less  $\beta$ -Gase inhibition.

#### *Preparation of Microsomes*

Tissues were homogenized in 0.25 M sucrose to give a 1:10 homogenate which was first centrifuged for 10 min at 10,000g and then the supernate was recentrifuged at 100,000g for 90 min to pellet the microsomes. Microsomes were washed and resuspended in 0.15 M KCl for the  $\beta$ -Gase assay (2).

#### *$\beta$ -Gase Assay*

Microsomal and serum  $\beta$ -Gase was assayed at pH 4.5 using a commercial kit (Sigma Chemical Co., St. Louis, MO) as modified (2). The incubation mixture also contained a 0.2  $\mu$ l/mg protein of Triton X-100. For bacterial  $\beta$ -Gase the assay pH was raised to 6.5; the remainder of the procedure was identical to microsomal preparation.  $\beta$ -Gase activity is expressed as modified Sigma units (MSU) per milligram protein or per milliliter serum. One MSU is defined as the activity of enzyme liberating 1.0  $\mu$ g of phenolphthalein from the glucuronide in 1 hr at 56°C.

#### *Determination of Glucaric Acid Content of Fruits and Vegetables*

Fruit and vegetable extracts were prepared by first heating 100 g of the material in 100 ml of distilled water for 30 min at 90°C, then homogenizing it in a Sorvall Omnimixer. The homogenate was centrifuged at 10,000g for 15 min and the supernate was filtered. Nonpolar substances were removed from the extract by three ether extractions and the aqueous phase was stored at -20°C until analysis.

The extracts were assayed for GL by the  $\beta$ -Gase inhibition assay of Colombi *et al.* (11), which in turn is a measure of glucaric acid content. This assay utilizes limpet  $\beta$ -Gase, with phenolphthalein mono $\beta$ -glucuronide as substrate (Sigma Chemical Co.). The free phenolphthalein formed in the reaction is estimated by alkalizing the reaction mixture (pH 11) and reading the absorbance at 550 nm. The limpet enzyme is particularly suited since its low pH optimum (pH 3.8) is near optimal for the equilibrium conversion of glucarate to glucarolactone. Prior to assay the samples and standards were boiled at pH 3.8 for 30 min to establish equilibrium among glucarate (33.3%), D-glucaro-1,4-lactone (33.3%), and D-glucaro-1,6-lactone (33.3%). Only the 1,4-lactone inhibits  $\beta$ -Gase (1). A standard curve was prepared by boiling known amounts of glucaric acid (potassium hydrogen salt; Sigma Chemical Co.) at pH 3.8 for 30 min before assay.

To obtain a second estimate of glucaric acid content, the plant extracts were purified by Dowex 1-X8 chromatography (12), prior to assay by the above procedure. A 2.0-ml aliquot of the extract was mixed with 2.0 ml of 0.1 M sodium borate and allowed to incubate at room temperature for 15 min to convert the glucarolactone to glucarate. The column was washed with 150 ml of 0.05 M sodium borate-0.02 M sodium sulfate, then the glucarate was eluted with 50 ml of 0.05 M sodium borate-0.1 M sodium sulfate. The flow rate was 50 ml per hour and 5.0-ml fractions were collected. The recovery of glucaric acid varied from 91.4 to 109%. The location of the glucarate in the eluate was determined by the

$\beta$ -Gase inhibition assay described above, but using a sample aliquot of 2.0 rather than 0.2 ml. The total GL in the eluate was obtained by summing all fractions.

The presence of glucarate in the Dowex column eluate was confirmed by HPLC using a Bio-Rad HPLC system equipped with a Bio-Rad HPX-87 H organic acid column (Bio-Rad Labs, Richmond, CA). Isocratic elution was done with 0.01 N sulfuric acid containing 10% acetonitrile and the eluate was monitored at 210 nm. The retention time of glucarate was determined with the authentic compound.

#### Statistical Evaluation

Statistical comparison between sample means was made by the two-tailed group Student's *t* Test. The level of significance in all cases was considered at  $P < 0.05$ .

### RESULTS

The effect of acute treatment of CaG on  $\beta$ -Gase activity is given in Table 1. A single dose (4.5 mmole/kg body wt) of CaG caused 57, 44, 37, and 39% inhibition of  $\beta$ -Gase activity in serum and microsomes from liver, lung, and intestine, respectively. All these inhibitions are statistically significant ( $P < 0.05$ ).

The temporal change in  $\beta$ -Gase activity in the serum from control and 4% CaG-supplemented diet rats during the 24-hr period is given in Fig. 1. The highest serum  $\beta$ -Gase activity in the control group was observed at 2:00 PM and the lowest at 10:00 AM. Only serum  $\beta$ -Gase activity at 12:00 noon and 2:00 PM were significantly different from other time periods. In the CaG-supplemented group, maximum inhibition was also observed at 12:00 noon and 2:00 PM. In general,  $\beta$ -Gase activity was decreased in the afternoons in the CaG-supplemented group.

The hepatic microsomal  $\beta$ -Gase activity in control and CaG-supplemented rats is given in Fig. 2. Maximum  $\beta$ -Gase activity in hepatic microsomes was observed at 2:00 AM and lowest at 2:00 PM.  $\beta$ -Gase activity was in general higher in the mornings and lower during the afternoons. Inhibition of  $\beta$ -Gase activity in CaG-supplemented group was also observed during early mornings. A similar pattern was also observed in intestinal microsomes (Fig. 3). However, a secondary

TABLE I  
Microsomal  $\beta$ -Gase in Fischer Female Rats after Receiving a Single Dose of Calcium Glucarate by Gavage

Treatment	$\beta$ -Gase activity <sup>a</sup>			
	Serum (MSU/ml)	Liver microsomes (MSU/mg protein)	Lung microsomes (MSU/mg protein)	GI tract microsomes (MSU/mg protein)
1. Control	59.7 $\pm$ 2.8	255.12 $\pm$ 13.88*	99.83 $\pm$ 9.01	51.89 $\pm$ 1.43
2. Treatment	25.8 $\pm$ 1.6*	142.82 $\pm$ 13.88*	62.83 $\pm$ 5.93*	31.85 $\pm$ 3.61*

Note. Dosage, 4.5 mmole/kg body wt; MSU, modified Sigma unit; GI microsomes, microsomes prepared from mucosal cells of entire intestinal tract.

<sup>a</sup> Data represent the means  $\pm$  SD.

\* Statistically significant difference ( $P < 0.05$ ) from control rats.

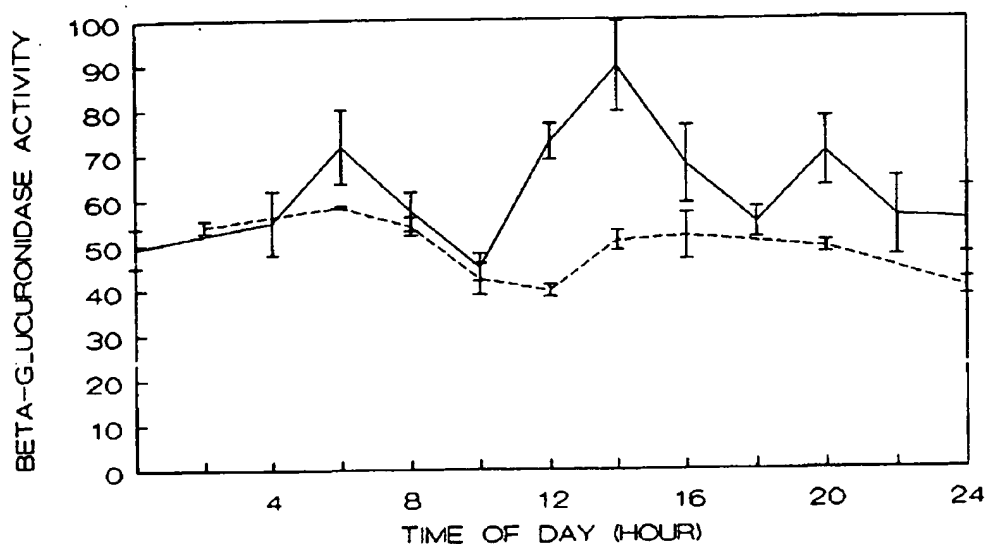


Fig. 1. Temporal changes in  $\beta$ -Gase activity in the serum (MSU/ml). The measurements were made over a 24-hr period. Values represent means  $\pm$  SD in control (—) and CaG-treated (---) rats. MSU, modified Sigma unit (see Materials and Methods for definition).

depression in  $\beta$ -Gase activity was also observed in intestinal microsomes around 4:00 PM. The latter may be due to direct absorption from the intestinal contents of the residual glucarate not absorbed from the stomach and duodenum.

The effect of dietary CaG on intestinal bacterial  $\beta$ -Gase is given in Table 2.

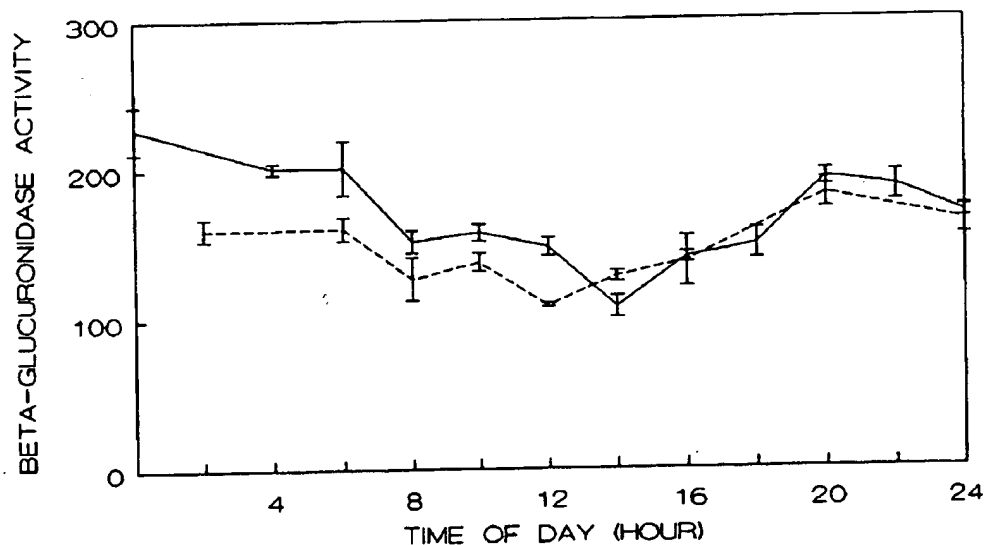


Fig. 2. Temporal changes in  $\beta$ -Gase activity in the liver microsomes (MSU/mg protein). The measurements were made over a 24-hr period. Values represent means  $\pm$  SD in control (—) and CaG-treated (---) rats. MSU, modified Sigma unit (see Materials and Methods for definition).

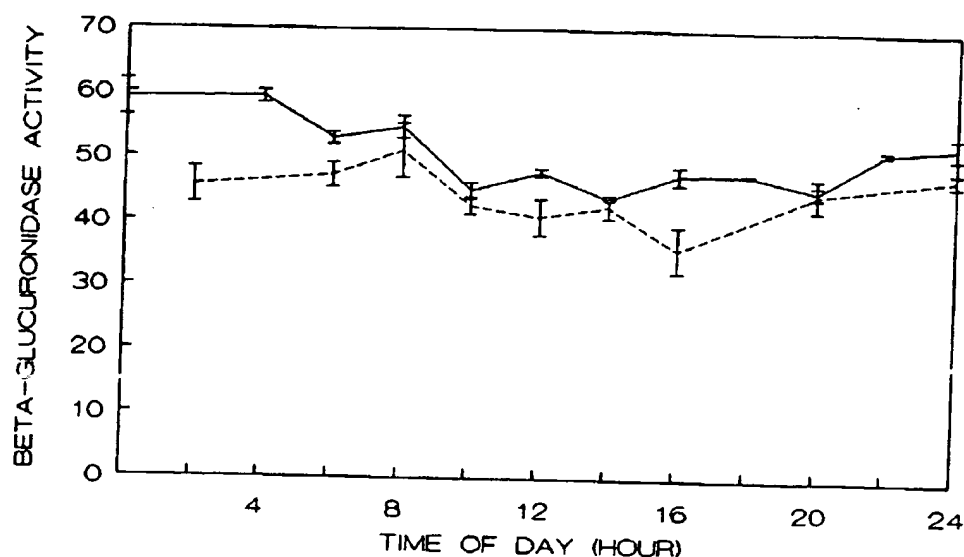


FIG. 3. Temporal changes in  $\beta$ -Gase activity in the intestinal microsomes (MSU/mg protein). The measurements were made over a 24-hr period. Values represent means  $\pm$  SD in control (—) and CaG-treated (---) rats. MSU, modified Sigma unit (see Materials and Methods for definition).

Animals on the 4% CaG-supplemented diet had 70 and 54% decreased  $\beta$ -Gase activities in the intestinal contents with bacterial enzyme activity from proximal (small intestine) to distal (colon) segments, respectively. This decrease in  $\beta$ -Gase activity is statistically significant ( $P < 0.05$ ).

Fruit and vegetable extracts were analyzed for GL which is a measure of glucarate content, both before and after chromatographic fractionation of Dowex 1-X8. In all cases the knowns and unknowns containing glucarate were boiled at pH 3.8 for 30 min to establish the equilibrium between glucarate and the lactones before assay with  $\beta$ -Gase. The results of these analyses are summarized in Table 3. Low but significant concentrations of glucaric acid were detected in all plant extracts examined. Glucarate content obtained from the crude extracts and Dowex semipurified fractions were in reasonable agreement for the carrot and orange, but other substances appeared to be present in the crude extracts

TABLE 2  
Effect of Calcium Glucarate (CaG) on Intestinal Bacterial  $\beta$ -Gase

Treatment	Intestinal $\beta$ -Gase activity <sup>a</sup> (MSU/Mg mucosal protein)	
	Proximal	Distal
Control	15.6 $\pm$ 1.53	12.7 $\pm$ 0.65
4% CaG-fed	4.5 $\pm$ 0.93*	5.8 $\pm$ 1.3*

Note. MSU, modified Sigma unit; Proximal, small intestine; Distal, colon.

<sup>a</sup> Data represent the means  $\pm$  SD.

\* Significantly different from control ( $P < 0.05$ ).

TABLE 3  
Glucaric Acid Content of Selected Fruits and Vegetables

Fruit or Vegetable (portion)	Glucarate (mg/100 g) <sup>a</sup>	
	Crude extract	Dowex 1-X8 purified
Orange (peeled)	4.63 ± 0.58	4.53 ± 0.62
Spinach (leaf)	1.58 ± 0.35	2.66 ± 0.18
Apples (whole)	1.57 ± 0.09	2.42 ± 0.15
Carrot (whole)	2.45 ± 0.32	2.32 ± 0.13
Alfalfa sprouts (whole)	0.82 ± 0.06	2.30 ± 0.16
Potatoes (peeled)	0.74 ± 0.08	1.73 ± 0.21
Broccoli (whole)	1.52 ± 0.25	1.12 ± 0.05

<sup>a</sup> Data represent the means ± SD of three separate determinations.

of potato, alfalfa sprouts, apple, and spinach which interfered slightly with the assay. Based on the semipurified fractions, the highest glucarate content was in oranges, followed by spinach leaves, apples, carrots, alfalfa sprouts, potatoes, and broccoli. Oranges, with the highest content, provide 4.53 mg of glucarate per 100 g of the fruit.

The presence of glucarate in the inhibitory fractions obtained by chromatographing the extract of oranges on the Dowex column was confirmed by HPLC analyses. A peak corresponding to glucarate was detected with a retention time of 12.6 min (Fig. 4). As expected, this peak was reduced in size upon boiling the fractions due to the conversion of two-thirds of the glucarate to the lactones.

## DISCUSSION

UDPGT, the enzyme which catalyzes phase II conjugation reactions, appears to consist of a number of species with different specificities and is firmly integrated into the endoplasmic reticulum (13). The deglucuronidation reaction is catalyzed by  $\beta$ -Gase, one-third of which is also present in the endoplasmic reticulum, with about two-thirds in the lysosomes (14).  $\beta$ -Gase activity is also present in the serum (2). The microsomal  $\beta$ -Gase is bound to the protein egasin and differs from the lysosomal  $\beta$ -Gase only in the carbohydrate moiety. In fact, both lysosomal and microsomal enzymes are coded by the same gene (14). GL, which is derived from dietary glucarate in this study, has been shown to be accessible to and to inhibit only microsomal enzymes (2), consistent with the very limited uptake of glucaric acid and other charged molecules by lysosomes (15). CaG following equilibrium has been found to have  $\beta$ -Gase inhibition constant  $K_i$  of 1.6  $\mu$ M (1). UDPGT is not affected by these treatments (2). Thus only microsomal  $\beta$ -Gase activities were evaluated in the present study.

Lower  $\beta$ -Gase activity caused by CaG-supplemented diet in liver microsomes was observed between 8 AM and 12 noon, which is consistent with the absorption of glucarate and lactone from the intestine and with nocturnal eating habits of the rodents. Thus inhibition of  $\beta$ -Gase by CaG (or GL) diminishes the deglucuronidation, and thereby may increase net glucuronidation. Such increased net glucuronidation could lead to increased excretion of toxins, drugs, steroid



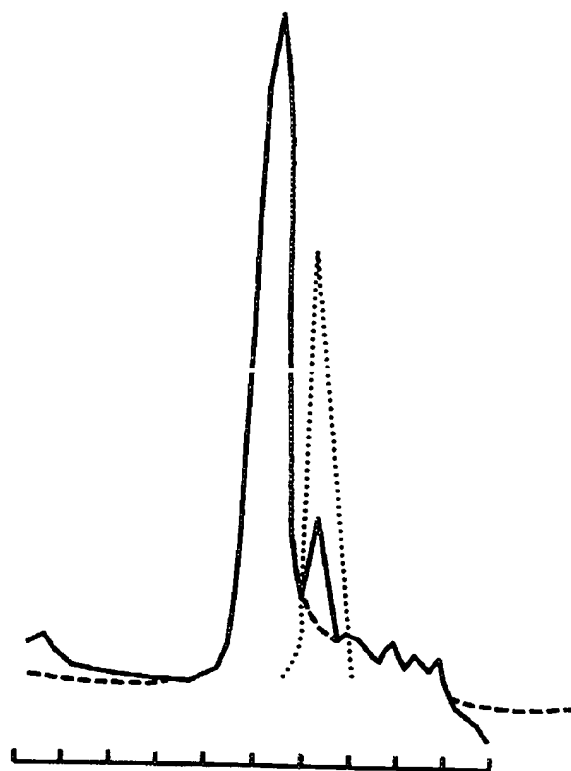


FIG. 4. HPLC analysis of fractions from Dowex 1-X8 column which were inhibitory toward  $\beta$ -Gase. Combined active fractions before (—) and after (---) boiling. The profile after addition of pure glucarate is also shown (···). Each division on the abscissa represents a retention time of 2 min. The ordinate represents the relative absorbance of the eluate at 210 nm.

hormones, and other compounds subject to glucuronidation. Dietary CaG was previously found to protect against 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced mammary carcinogenesis in rats (3), urethane-induced hepatocarcinogenesis (5), and DMBA-induced and tetradecanoylphorbolacetate-promoted skin carcinogenesis in mice (6). Takada *et al.* (10) have similarly demonstrated a protective effect of a synthetic  $\beta$ -Gase inhibitor in azoxymethane-induced rat colon carcinogenesis. Increased excretion of bilirubin due to increased net glucuronidation as a result of inhibition of  $\beta$ -Gase has been demonstrated (2).

Further support for the action of dietary glucarate through inhibition of the  $\beta$ -Gase comes from the synergistic interaction observed between glucarate and retinoids in the chemoprevention of cancer in experimental animals (7). This synergism was attributed to net increased glucuronidation of retinoids through inhibition of  $\beta$ -Gase (7). The basis for this proposed mechanism was the evidence by other investigators for the formation of glucuronides of natural and synthetic retinoids which are biologically active and in some cases determined to be less cytotoxic than the parent compound (16–18). Increased net formation of bilirubin

glucuronide, a result of inhibition of  $\beta$ -Gase, has been shown for rats fed 128 mmole/kg diet of CaG (2).

Besides increasing net glucuronidation of substrates by inhibiting  $\beta$ -Gase in serum and tissues, dietary glucarate may also reduce enterohepatic circulation by inhibiting  $\beta$ -Gase in the intestinal contents. The results of this investigation do show that dietary glucarate markedly inhibits  $\beta$ -Gase in the intestinal contents in addition to  $\beta$ -Gase in the intestinal mucosa. One or both of these effects may be involved in the inhibition of colon carcinogenesis by CaG in rats (19). The enterohepatic circulation of many drugs, toxins (including carcinogens and promoters), and endogenous steroids, may also be modified by dietary glucarate.

In view of the potential effects of dietary glucarate on net glucuronidation and excretion of endogenous and exogenous compounds, as well as the enterohepatic circulation of those excreted in the bile, it was of interest to evaluate glucarate levels in fruits and vegetables. Of the seven fruits and vegetables assayed, oranges contained the highest level (4.5 mg/100 g) of glucarate. Biologically effective concentrations of glucarate alone probably cannot be achieved by consuming fruits and vegetables. Comparable amounts may, however, interact synergistically with retinoids and other biologically active compounds (cf. (7)). CaG at the physiological level may not produce a significant effect on  $\beta$ -Gase and/or net glucuronidation but dietary supplementation or pharmacological treatment with CaG influences net glucuronidation. Although CaG is not a common food additive, it is used clinically as a stabilizer in supersaturated calcium gluconate solutions utilized for the rapid adjustment of calcium ion (20). CaG also produces no toxic symptoms in rats with up to 10% dietary supplementation (unpublished observation). In view of the chemoprotective activity of the CaG (2-7,19) and its potential impact on the net glucuronidation of drugs, toxins, and hormones as a result of  $\beta$ -Gase inhibition, this compound warrants further investigation.

#### SUMMARY

Glucarate is normally present in tissues and body fluids and is in equilibrium with D-glucaro-1,4-lactone, a natural inhibitor of  $\beta$ -glucuronidase activity. Dietary calcium glucarate, a sustained-release form of glucarate, elevates the blood level of D-glucaro-1,4-lactone which suppresses blood and tissue  $\beta$ -glucuronidase activity. A single dose of CaG (4.5 mmole/kg body weight) inhibited  $\beta$ -glucuronidase activity in serum and liver, lung, and intestinal microsomes by 57, 44, 37, and 39%, respectively. A chronic administration of calcium glucarate (4% in diet) also decreased  $\beta$ -glucuronidase activity in intestinal and liver microsomes. Maximal inhibition of  $\beta$ -glucuronidase activity in serum was observed from 12 noon to 2:00 PM. In contrast, maximum inhibition of  $\beta$ -glucuronidase activity in intestinal and liver microsomes occurred during mornings, although a secondary depression in intestinal microsomes also occurred around 4 PM. A 4% calcium glucarate supplemented diet also inhibited  $\beta$ -glucuronidase activity by 70% and 54%, of the bacterial flora obtained from proximal (small intestine) and distal (colon) segments of intestine, respectively.

Due to the potential effect of dietary glucarate on net glucuronidation and on other metabolic pathways, glucaric acid levels in various foods were determined.

The glucaric acid content varied from a low of 1.12–1.73 mg/100 g for broccoli and potatoes to a high of 4.53 mg/100 g for oranges.

### ACKNOWLEDGMENTS

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